PB87-203063

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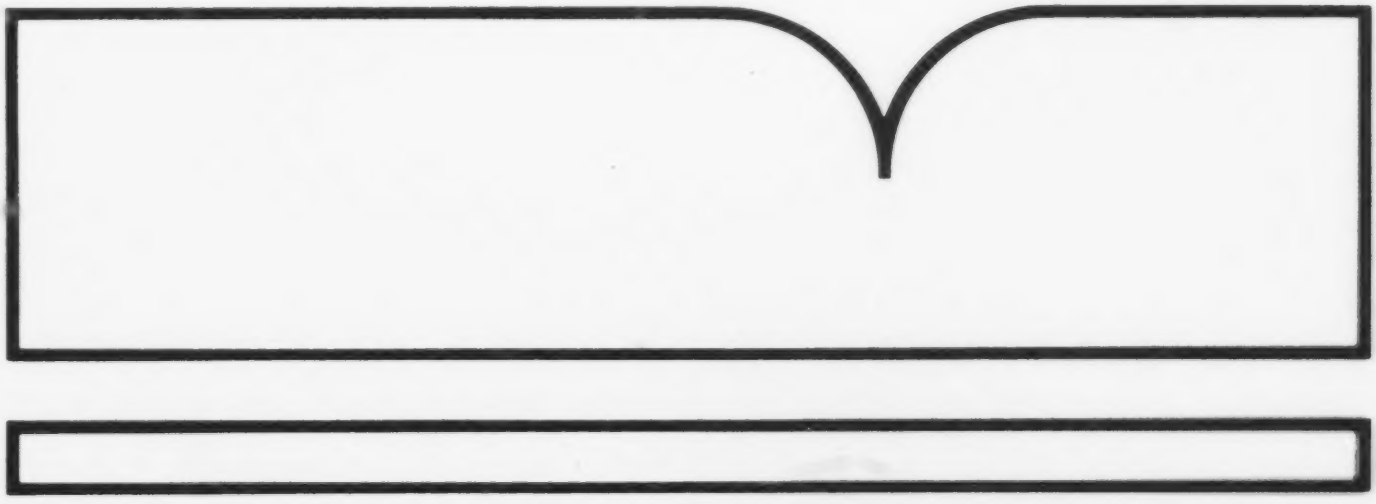
Research Triangle Park, NC

Jun 87



U.S. Department of Commerce
National Technical Information Service
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PB87-203063

EPA/600/D-87/209
June 1987

METHODOLOGY FOR EVALUATING POTENTIAL HUMAN HEALTH EFFECTS OF MICROORGANISMS THAT DEGRADE HAZARDOUS WASTES

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TECHNICAL REPORT DATA *(Please read Instructions on the reverse before completing)*		
1. REPORT NO. EPA/600/D-87/209	2.	3. RECIPIENT'S ACCESSION NO. PB87 203063/AS
4. TITLE AND SUBTITLE Methodology for Evaluating Potential Human Health Effects of Microorganisms that Degrade Hazardous Wastes		5. REPORT DATE June 1987 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) S.E. George*, Michael J. Kohan*, Debra B. Walsh**, and Larry D. Claxton*		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS *U.S. Environmental Protection Agency HERL, GTD, RTP, NC 27711 **Environmental Health Research and Testing RTP, NC 27709		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory, RTP Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA/600/11
15. SUPPLEMENTARY NOTES		

16. ABSTRACT

Microorganisms are being developed to environmentally degrade hazardous wastes. Before such organisms are deployed, methods need to be designed to monitor the organisms and waste by-products for potentially adverse human health and environmental effects. Initial work in our laboratory involved the study of the biological effects of a mutant microorganism that biodegrades polychlorinated biphenyl compounds (PCB's). We have designed several mouse model systems to examine the ability of these organisms to colonize the intestines of the mouse and compete with the resident intestinal floral. These include two 48 hour studies with and without ampicillin pretreatment, a 14 day study in conventional and metabolism cages, and examination of the fecal material. Mice were dosed by gavage with 10^3, 10^6, or 10^9 organisms and sacrificed at time intervals up to 48 hours following exposure. The hazardous waste degrading microorganisms, Pseudomonas aeruginosa, was recoverable on a selective medium in all methods evaluated. The normal intestinal flora was also monitored. This included enumeration of the lactose positive enteric microorganisms, total aerovic and anaerobic counts, and the obligately anaerobic, predominatly Gram-negative rod populations. Depending on the method utilized, an alteration in one or more of the normal flora was observed in a dose related manner.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
	UNCLASSIFIED	28
RELEASE TO PUBLIC	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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ABSTRACT

Microorganisms are being developed to environmentally degrade hazardous wastes. Before such organisms are deployed, methods need to be designed to monitor the organisms and waste by-products for potentially adverse human health and environmental effects. Initial work in our laboratory involved the study of the biological effects of a mutant microorganism that biodegrades polychlorinated biphenyl compounds (PCB's). We have designed several mouse model systems to examine the ability of these organisms to colonize the intestines of the mouse and compete with the resident intestinal flora. These include two 48 hour studies with and without ampicillin pretreatment, a 14 day study in conventional and metabolism cages, and examination of the fecal material. Mice were dosed by gavage with 10^3, 10^6, or 10^9 organisms and sacrificed at time intervals up to 48 hours following exposure. The hazardous waste degrading microorganisms, Pseudomonas aeruginosa, was recoverable on a selective medium in all methods evaluated. The normal intestinal flora was also monitored. This included enumeration of the lactose positive enteric microorganisms, total aerobic and anaerobic counts, and the obligately anaerobic, predominatly Gram-negative rod populations. Depending on the method utilized, an alteration in one or more of the normal flora was observed in a dose related manner.

INTRODUCTION

Microorganisms are being isolated and developed to biodegrade hazardous wastes such as polychlorobiphenyls (PCB)(18, 24), formaldehyde (18), chlorobenzoates (10, 4) and pesticides (21, 20). The ability of microorganisms to detoxify the target compounds is being enhanced through mutagenicity or recombinant DNA techniques. The ultimate goal is to apply these organisms to a site contaminated with an environmentally hazardous compound and metabolize it to a nonhazardous state, in situ. The need to transport the compound to a central location such as a landfill or incinerator would be unnecessary, thus eliminating the associated risks.

Very little research has been reported on the potential human health effects associated with these biodegradative microorganisms. The proposed release of these bacteria constructed through the use of recombinant DNA techniques into the environment has received national attention (6, 7, 9). Federal and state agencies now regulate the environmental application of these bacterial strains (17, 19). The purpose of this study is to propose and evaluate methods that will examine the potential human health effects of these recombinant, mutant, and naturally occurring microorganisms used to detoxify hazardous environmental chemicals. The methods presented in this study examine the potential of these biodegradative microbes to colonize the intestines of mice and determine how they compete with the normal flora. An alteration of the normal flora may indicate a

change in the intestinal metabolism that could have an effect on digestion, absorption, and the overall physiology of the host. The results reported depict the competition and colonization potential of a PCB degrading microorganism, P. aeruginosa, that was isolated from a commercially available product for PCB degradation. Manuscripts submitted and in preparation describe this research in greater detail.

MATERIALS AND METHODS

Chemicals. All chemicals utilized in this study were obtained commercially and of reagent grade. Vancomycin HCl, kanamycin sulfate, ampicillin, hemin, and vitamin K_1 were purchased from Sigma Chemical Company, St. Louis, MO. Mercuric chloride was obtained from Mallinckrodt, Paris, KY.

Media. P. aeruginosa strain BC16 was enumerated on Pseudomonas isolation agar (Difco, Detroit, MI) containing mercuric chloride ($HgCl_2$) (30 µg/ml). MacConkey agar (Difco) was prepared according to the manufacturer's direction. Brucella blood agar (BBA) was prepared as follows: Brucella agar (BBL Microbiology Systems, Cockeysville, MD) as directed, 5% (v/v) laked, defibrinated sheep blood (Environmental Diagnostics, Burlington, NC), vitamin K_1 (1.0 µg/ml) and hemin (5 µg/ml)(26,11). Where indicated, vancomycin HCL (7.5 µg/ml) and kanamycin (75 µg/ml) were added to BBA after autoclaving. Yeast extract-tryptone (YT) (15) medium without added NaCl was used as culture medium where indicated.

Bacterial strains. P. aeruginosa was isolated from Bi-Chem® 1006 PB (Sybron Chemicals, Inc., Salem, VA) as directed by the manufacturer. Bacterial strains in this product were designed to biodegrade polychlorinated biphenyl compounds. Identification was confirmed by Gram staining and using the API20E® method (Analtab Products, Plainview, NY).

Culture conditions. All incubations for bacterial growth were done at 37°C. Inocula for dosing was prepared by sedimenting an overnight 25 ml YT culture of P. aeruginosa . The cells were resuspended in 5 ml Dulbeccos phosphate buffered saline (PBS) (GIBCO Laboratories, Chagrin

Falls, OH) prior to animal dosing. All other incubations were done aerobically for 48 h or anaerobically for 72 h.

Animal studies. Sixty-day old strain CD-1 male mice (Charles River Laboratories, Raleigh, NC or Kingston, NY) were used in the studies. Animals were given sterilized food (Sterilizable Rodent Blox®, Continental Grain Company, Chicago, IL) and water ad libitum. Animals were housed in cages with wood shaving bedding unless otherwise indicated. When metabolism cages (Nalge Company, Rochester, NY) were employed, animals were fed sterile pelletized chow (Dustless Pellets-45 mg R.C., Bioserv Inc., Frenchtown, NJ) and water ad libitum. Animals were administered 0, 10^3, 10^6, or 10^9 CFU/ml of P. aeruginosa by gavage. At time intervals (0, 3, 6, 12, 24, 48 for the 48 h study, 14 days for the metabolism cage study, and 3, 12, 24, and 48 h for the ampicillin pretreatment 48 h study) the animals were sacrificed and the intestines surgically removed and immediately placed into 5 ml reduced PBS. The intestines were homogenized using a Polytron® tissue homogenizer (Brinkman Instruments, Westbury, NY) under a constant stream of nitrogen and then placed into an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. Serial dilutions of the homogenate were made in reduced PBS and all anaerobic plating and incubation was done in the chamber. Aerobic plating was done aerobically on selective media (Figure 1).

Where indicated, feces were collected from regular cages on a daily basis (1,2, and 3 days) or metabolism cages at 0, 1, 2, 4, 6, 8, 10, 12, and 14 days after dosing. Approximately 1.0 g of fecal material was

placed into 5 ml PBS, vortexed, and the dosed microorganisms selected for on Pseudomonas isolation agar containing $HgCl_2$ (30 µg/ml).

Three independent animal studies were performed using the above protocol. The first examined the survival and competition potential of the dosed microorganism over a period of 48 h. Results are an average of values enumerated from four animals per dose-time combination. The second study compared the flora from animals housed in regular (five animals per dose-time combination) or metabolism (five or ten animals per dose-time combination as indicated) cages for 14 days after dosing. In the third study, animals were pretreated with 5 mg of ampicillin on days 1 and 2 then 1 mg of ampicillin on day 3 prior to dosing with the microorganism (four animals per dose-time combination). A general outline for the animal studies is shown in Figure 1.

Statistical analysis. Analyses of variance and T-tests were performed using the RS/1 statistical software package (BBN Software Products Corporation, Irving, Texas) on an IBM-AT personal computer.

RESULTS

Recovery of P. aeruginosa in the feces. Fecal material was collected from the cages over a three day period. Recovery of the microorganism was examined by selection on Pseudomonas isolation agar containing $HgCl_2$. The results are shown in Table 1. P. aeruginosa was recovered only from animals at the higher dose.

A more in depth study was done in metabolism cages, using only the high dose, 10^9 CFU, where the fecal material was collected and the organisms enumerated. P. aeruginosa was recovered from the fecal material for only two days after dosing (Table 1).

Survival of P. aeruginosa in the gastrointestinal tract. A more quantitative assay for the survival of the dosed microorganism was needed so the intestines were analyzed for recovery and competition. Three separate studies were performed to look at the competition of the PCB degrading microorganism in the mouse intestines: 1) enumeration over 48 hours, 2) ampicillin pretreatment followed by enumeration over 48 hours, and 3) enumeration after 14 days in regular and metabolism cages. In both 48 hour studies, the survival of the organism was similar with counts in the 10^1 CFU/g intestines range for animals administered 10^9 CFU (Table 2). Animals dosed with 10^3 CFU yielded $<10^1$ CFU/g intestines after 48 hours in both ampicillin treated and untreated animals.

In the 14 day study, P. aeruginosa appeared to colonize the gastrointestinal tract in animals dosed with 10^9 CFU yielding 4.0×10^4 CFU/g intestines (Table 2). Animals housed in metabolism cages, where fecal material was not in contact with the animals, yielded $<10^1$ CFU/g intestines.

Effect of P. aeruginosa on the normal intestinal flora. Four populations of normal flora were monitored in the 48 hour and 14 day studies. They included 1) lactose positive enteric organisms, 2) total aerobic counts, 3) total obligately anaerobic predominantly Gram-negative rods, and 4) total anaerobic counts.

In the 48 hour study, the lactose positive counts ranged from 5.0×10^3 to 3.8×10^5 CFU/g intestines. Results from the 14 day study gave number of similar magnitude. Ampicillin pretreatment markedly increasd these counts yielding a range from 5.4×10^7 to 4.5×10^9 CFU/ g intestines. The results are tabulated in Table 3.

The total aerobic count, which included aerobes and facultative anaerobes was also increased in animals pretreated with ampicillin. The counts are shown in Table 4. Ampicillin pretreatment selected for several different species including Enterobacter cloacae (predominant), Proteus vulgaris, and Lactobacillus spp. Though numbers were lower in the animals not pretreated with ampicillin, a more diverse group of organisms was observed and rarely were E. cloacae or P. vulgaris observed. Values in the 10^7-10^8 CFU/g intestines range were observed in the animals without ampicillin treatment whereas those that received ampicillin had counts in the 10^7-10^9 CFU/g intestines range.

Ampicillin pretreatment greatly reduced the obligately anaerobic Gram-negative rod counts (Table 3). These organisms were selected for by resistance to the antibiotics kanamycin and vancomycin. Included in this group were Bacteroides spp. and Fusobacterium spp. (8). Ampicillin

treatment reduced the counts from 10^7-10^8 CFU/g intestines in the 48 hour and 14 day studies to 10^1-10^4 CFU/g intestines in the ampicillin pretreatment study. Results are shown in Table 5.

The total anaerobic counts from mice dosed with P. aeruginosa are tabulated in Table 6. Included in this count were facultative and strict anaerobes. Facultative organisms included Streptococcus spp., Staphylococcus spp., Lactobacillus spp., and organisms from the family Enterobacteriaceae (2). Strict anaerobes included the Clostridia, Eubacteria, Bacteroides spp., and the Fusobacteria as well as others (8, 2, 16).

In the 48 hour study, counts range from 2.2×10^8 to 8.7×10^8 CFU/g intestines. The 14 day study in regular and metabolism cages showed similar values. Again, ampicillin increased the observed counts. Values ranged form a low of 2.7×10^4 to a high of 2.2×10^9 CFU/g intestines. The flora enumerated were similar to that observed in the total aerobic counts.

In order to determine whether the presence of P. aeruginosa caused an alteration in the normal murine intestinal flora, statistical analyses were performed on the data collected in Tables 3-6. P-values are reported in Table 7.

DISCUSSION

This study was designed to develop and evaluate methods to determine some of the indirect adverse human health effects potentially associated with microorganisms that degrade hazardous wastes. Four different studies were proposed, three of which involved enumeration of the biodegrading microorganisms from the gastrointestinal tract and one study that examined the fecal material for the organism's presence. Further effects on the normal flora were investigated. They included enumeration of 1) the enteric lactose positive microorgnisms, 2) the total aerobic counts, 3) obligately anaerobic Gram-negative rod counts, and 4) the total anaerobic counts.

The initial study involved the examination of the mouse fecal material for the presence of the dosed biodegrading microorganism. This method was utilized by Levy and Marshall (13) and Levy et al. (14) to determine colonization of recombinant Escherichia coli strain in mice. Similar studies were done in human volunteers (1, 14, 22, 23). The results were not very promising with recovery being observed in regular cages only at the highest dose, 10^9 CFU (Table 1). Further studies in metabolism cages extended the recovery time to two days at the same dose. Several questions arose such as whether oxygen or dessication had an effect on the microbial content. In order to avoid these potential problems, the mouse intestines were examined.

None of the mice dosed by gavage with P. aeruginosa appeared unhealthy during these studies. No diarrhea was observed and animal weights did

not decrease. No abnormal pathology was observed. Therefore, all of these methods were able to evaluate the direct health effects on mice such as morbidity or mortality.

In the 48 hour study, P. aeruginosa was recoverable from the mouse intestines at all three doses. By the end of 48 hours, organisms were countable on the selective medium.

Ampicillin pretreatment was incorporated into the study in order to alter the normal flora and enhance the survival of the biodegradative microbe, which was resistant to ampicillin. Cohen et al. (5) and Laux et al. (12) used streptomycin to select for coloniation by E. coli derivatives. Ampicillin pretreatment did not appear to have a selective effect on the survival of strain BC16 at 48 hours. Survival counts were similar for both the experiments (Table 2). An increase in survival at the other time points was noted in ampicillin treated animals.

A difference in normal flora counts was observed in the two 48 hour experiments. An increase in total counts was observed for the ampicillin treated animals in all populations monitored with the exception of the obligately anaerobic Gram-negative rods (Table 3-6). Antibiotics have been reported to alter the normal flora in the gastrointestinal tract populations (3,25). Ampicillin treatment decreased those counts by a least four orders of magnitude (Table 5). The increase in the other counts was due to an increase in the E. cloacae population. This organism is facultative so therefore can grow on MacConkey agar and Brucella blood agar both anaerobically and aerobically.

The resulting alterations in the normal flora between the two 48 hour studies differed. A dose effect ($p<0.05$) was observed for the lactose positive and obligately anaerobic Gram-negative rod counts in animals without ampicillin pretreatment (Table 7). The total anaerobic population counts were affected ($p<0.1$) in the ampicillin treatment experiment (Table 7).

Survival and alteration of the intestinal flora was also examined at the end of 14 days in animals housed in regular and metabolism cages. This provided results from a longer term of study. P. aeruginosa was recovered (4.0×10^4 CFU/g intestines) 14 days after dosing in animals housed in regular cages (Table 2). The results varied from those observed in animals housed in metabolism cages. Mice are copraphagic and the observed results were probably due to this phenomenum. Again the alterations in the normal flora were different. The obligately anaerobic Gram-negative rod and total anaerobic populations showed a significant difference ($p<0.1$) upon exposure to P. aeruginosa (Table 7). Only the total aerobic populations were affected ($p<0.1$) when mice were housed in regular cages.

The comparison of intestinal flora from animals housed in regular or metabolism cages provided a good model to compare repeated exposure versus a single dose. The organisms that were excreted were re-administered through this copraphagic behavior. Because all fecal material was separated from the animals in the metabolism cages, no re-dosing could occur through this route.

The 48 hour study provided a short term method for examining the fate of the biodegrading microorganism and its effect on the normal flora in both conventional mice as well as ones whose normal flora had been altered. The ampicillin treatment may give researchers insight into how compromized humans (those on antibiotic therapy or immunosuppressant drugs) may be effected upon exposure to this class of microorganisms.

These studies provide methods to examine the colonization and competition potential of microorganisms that degrade hazardous wastes. If the normal flora are altered due to the presence of these strains, then the effect on intestinal flora metabolism, absorbtion, and the host's physiology need to be considered. In vitro methods are currently being developed to investigate colonization, competition and metabolic changes associated with exposure to the biodegraders using both mouse and human intestinal flora.

ACKNOWLEDGMENTS

We are grateful to Ms. Angalena Monroe for her technical assistance in the laboratory and with the data entry.

We would also like to thank Sybron Chemicals, Inc for supplying our laboratory with a sample of Bi-Chem® 1006 PB.

The research described in this article has been funded wholly or in part by the Health Effects Research Laboratory, U. S. Environmental Protection Agency. Although this article has been reviewed by the Health Effects Research Laboratory, U. S. Environmental Protection Agency, Research Triangle Park, NC, it does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

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Table 1. Recovery of P. aeruginosa from mouse fecal material.[a]

	Time(days)	0	10^3	10^6	10^9
Regular Cages	1	-[b]	-	-	+
	2	-	-	-	-
	3	-	-	-	-
Metabolism Cages	0	-	nd[c]	nd	-
	1	-	nd	nd	+
	2	-	nd	nd	+
	3	-	nd	nd	-
	4	-	nd	nd	-
	6	-	nd	nd	-
	8	-	nd	nd	-
	10	-	nd	nd	-
	12	-	nd	nd	-
	14	-	nd	nd	-

[a] Mice were dosed with P. aeruginosa and at designated time intervals, approximately 1.0 gram of fecal material was collected and placed into 5.0 ml PBS. A 0.1 ml aliquot was plated onto Pseudomonas isolation agar containing $HgCl_2$, incubated 24 hours, and the organism enumerated.

[b] (-), no organism detected; (+), growth of dosed strain was evident.

[c] not determined

Table 2. Survival of P. aeruginosa in mouse intestines.[a]

		Dose (CFU/animal)			
	Time(h)	0	10^3	10^6	10^9
48 hour study	0	$<10^1$[b]	6.1×10^0	1.1×10^2	1.1×10^6
	3	$<10^1$	2.4×10^0	1.3×10^3	5.0×10^5
	6	$<10^1$	7.5×10^0	2.0×10^2	6.0×10^5
	12	$<10^1$	$<10^1$	2.9×10^0	1.7×10^4
	24	$<10^1$	$<10^1$	4.6×10^{-1}	1.8×10^1
	48	$<10^1$	2.6×10^0	2.0×10^1	1.6×10^1
48 hour study ampicillin pretreatment	3	$<10^1$	1.1×10^1	n.d.[d]	4.6×10^5
	12	$<10^1$	$<10^1$	n.d.	3.9×10^6
	24	$<10^1$	$<10^1$	n.d.	2.0×10^5
	48	$<10^1$	$<10^1$	n.d.	2.1×10^1
	Time(d)				
14 Day study[e]:					
regular cages	14	$<10^1$	n.d.	n.d.	4.0×10^4
metabolism cages	14	$<10^1$	n.d.	n.d.	$<10^1$

[a] Mice were dosed with P. aeruginosa. At designated time intervals, animals were sacrificed, the intestines removed and homogenized under N_2 gas in 5 ml reduced PBS. Homogenates were then placed into an anaerobic chamber and dilutions were made in PBS. A 0.1 ml. aliquot of the diluted material was used to inoculate Pseudomonas isolation agar containing $HgCl_2$. Plates were incubated 24-48 hours at 37°C. Counts are an average from 4 animals unless stated otherwise.

[b] No colonies were detectable on the enumeration medium.

[c] Animals were pretreated with 5 mg ampicillin (2 days) followed by 1 mg ampicillin (2 days) prior to dosing with P. aeruginosa.

[d] Not determined

e Counts are an average from 5 animals.

Table 3. Total lactose positive counts in P. aeruginosa dosed mice: enumeration from an intestinal homogenate.[a]

		Dose (CFU) x 10^4			
	Time	0	10^3	10^6	10^9
	(h)	CFU x 10^4			
48 hour study	0	37.7[b]	1.1[b]	1.8[b]	3.7[b]
	3	6.1	2.4	0.5	2.4
	6	1.2	10.1	5.5	12.8
	12	1.9	0.5	0.8	1.4
	24	5.9	3.6	4.1	2.3
	48	0.5	1.5	0.6	7.1
	(h)	CFU x 10^7			
48 hour study ampicillin pretreatment	3	156.1	67.9	n.d.[c]	184.3
	12	110.8	86.7	n.d.	95.9
	24	449.7	5.4	n.d.	202.3
	48	273.1	189.8	n.d.	337.9
	(days)	CFU x 10^3			
14 day study					
metabolism	14	23.0[d]	n.d.	n.d.	14.0[d]
regular	14	1.0[e]	n.d.	n.d.	0.3[e]

a Mice were dosed with 0, 10^3, 10^6, or 10^9 CFU and sacrificed at indicated time intervals. The intestines were removed, homogenized, and dilutions of the homogenate prepared in PBS. A 0.1 ml. aliquot of the appropriate dilution was spread plated onto MacConkey Agar and plates were incubated 24-48 hours. Counts are an average of values from 4 animals unless otherwise indicated.

b Counts are an average of values from 2 animals.

c not determined

d Counts are an average of values from 10 animals.

e Counts are an average of values from 5 animals.

Table 4. Total aerobic counts in P.aeruginosa dosed mice: enumeration from an intestinal homogenate.[a]

		Dose			
	Time	0	10^3	10^6	10^9
	(h)	CFU x 10^7			
48 hour study	0	4.8[b]	6.0[b]	2.6[b]	15.0[b]
	3	5.8	23.4[b]	9.1	12.9
	6	2.9	4.5[b]	14.7	7.1
	12	1.5	2.2[b]	1.7	1.5
	24	9.8	12.9[b]	13.1	10.8
	48	7.3	17.2[b]	12.6	22.5
	(h)	CFU x 10^7			
48 hour study ampicillin pretreatment	3	200.1[b]	87.6[b]	n.d.	247.8[b]
	12	116.2[b]	96.4[b]	n.d.	107.0[b]
	24	786.1[b]	8.0[b]	n.d.	311.5[b]
	48	411.9[b]	240.8[b]	n.d.	529.1[b]
	(days)	CFU x 10^7			
14 day study					
metabolism	14	0.22[d]	n.d.	n.d.	0.23[d]
regular	14	0.78[e]	n.d.	n.d.	2.65[e]

a Mice were dosed with 0, 10^3, 10^6, or 10^9 CFU and sacrficed at indicated time intervals. The intestines were removed, homogenized, and dilutions of the homogenate prepared in PBS. A 0.1 ml. aliquot of the appropriate dilution was spread plated onto Brucella Blood Agar and plates were incubated 48 hours. Counts are an average of values from 4 animals unless otherwise indicated.

b Counts are an average of values from 2 animals.

c not determined

d Counts are an average of values from 10 animals.

e Counts are an average of a values from 5 animals.

Table 5. Total obligately anaerobic predomiantly Gram negative rod counts in P.aeruginosa dosed mice: enumeration from an intestinal homogenate.[a]

		Dose(CFU)			
	Time	0	10^3	10^6	10^9
	(h)	CFU x 10^8			
48 hour study	0	1.0[b]	3.3[b]	1.8[b]	2.0[b]
	3	4.7[b]	2.9[b]	1.8[b]	3.1[b]
	6	3.6[b]	3.7[b]	3.8[b]	4.5[b]
	12	0.9[b]	1.3[b]	1.7[b]	2.6
	24	2.0[b]	1.3[b]	2.0	2.4
	48	1.1[b]	1.1[b]	2.3	2.4
	(h)	CFU x 10^2			
48 hour study ampicillin pretreatment	3	>10^4	16.3	n.d.[c]	1.0
	12	35.8	0.5	n.d.	3.4
	24	180.2	10.4	n.d.	683.9
	48	>10^4	672.3	n.d.	663.6
	(d)	CFU x 10^7			
14 day study					
metabolism	14	11.08[d]	n.d.	n.d.	6.54[d]
regular	14	8.80[d]	n.d.	n.d.	13.88[d]

[a] Mice were dosed with 0, 10^3, 10^6, or 10^9 CFU and sacrificed at indicated time intervals. The intestines were removed, homogenized, and dilutions of the homogenate prepared in PBS. A 0.1 ml. aliquot of the appropriate dilution was spread plated onto Brucella Blood Agar supplemented with vancomycin and kanamycin. Counts are an average of values from 4 animals unless otherwise indicated.

[b] Counts are an average of values from 2 animals.

[c] not determined

[d] Counts are an average of values from 5 animals.

Table 6. Total anaerobic counts in P.aeruginosa dosed mice: enumeration from an intestinal homogenate.[a]

	Time	Dose			
	(h)	0	10^3	10^6	10^9
48 hour study	0	2.3[b]	7.9[b]	4.8[b]	4.0[b]
	3	6.9	6.0[b]	4.2	6.4
	6	6.2	8.7[b]	7.6	6.2
	12	2.2	2.4[b]	2.4	2.6
	24	2.6	2.7[b]	3.5	4.1
	48	3.4	3.5[b]	5.9	6.3
	(h)			CFU x 10^7	
48 hour study ampicillin pretreatment	3	131.8	2.7	n.d.[c]	203.4
	12	145.6	42.1	n.d.	117.6
	24	145.7	9.4	n.d.	105.9
	48	203.8	223.5	n.d.	192.6
	(d)			CFU x10^8	
14 day study					
metabolism	14	8.45[d]	n.d.	n.d.	3.25[5]
regular	14	5.14[d]	n.d.	n.d.	3.84[5]

a Mice were dosed with 0, 10^3, 10^6, or 10^9 CFU and sacrificed at indicated time intervals. The intestines were removed, homogenized, and dilutions of the homogenate prepared in PBS. A 0.1 ml. aliquot of the appropriate dilution was spread plated onto Brucella Blood Agar. Counts are an average of values from 4 animals unless otherwise incated.

b Counts are an average of values from 2 animals.

c not determined

d Counts are an average of values from 5 animals.

Table 7. P-values for the effects of P.aeruginosa dose on the normal flora.

		48 hour study[a]	ampicillin pretreatment 48 hour study[a]	14 day study[b] metabolism cages	14 day regular cages
Lactose positive colonies	Dose	0.012	0.271	0.615	0.338
	Time	0.002	0.451	n.d.[c]	n.d.
	Nonadditivity	0.000	0.757	n.d.	n.d.
Total aerobic colonies	Dose	0.292	0.270	0.234	0.100
	Time	0.044	0.374	n.d.	n.d.
	Nonadditivity	0.866	0.684	n.d.	n.d.
Obligately anaerobic Gram-negative rods	Dose	0.013	n.d.	0.027	0.236
	Time	0.000	n.d.	n.d.	n.d.
	Nonadditivity	0.347	n.d.	n.d.	n.d.
Total anaerobic colonies	Dose	0.292	0.073	0.063	0.338
	Time	0.044	0.079	n.d.	n.d.
	Nonadditivity	0.866	0.513	n.d.	n.d.

[a] P-values from a two-way analysis of variance of the data obtained from enumeration of the normal intestinal flora.

b P-values from a T-test of the data obtained from enumeration of the normal intestinal flora.

c not determined

Figure 1. Protocol for animal study

Dose strain CD-1 60 day old male mice with microorganisms

↓

Sacrifice animals

↓

Remove intestines and homogenize under N_2

↓

Place homogenate into anaerobic chamber and prepare dilutions

↓

Plate 0.1 ml homogenate dilution onto selective media

Anaerobic media	Aerobic media
• Brucella laked blood agar	• *Pseudomonas* isolation agar
• Brucella laked blood agar + vancomycin and kanamycin	• MacConkey agar
	• Brucella laked blood agar